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Published in:
Molecular Nutrition and Food Research

DOI:
10.1002/mnfr.202000515

Publication date:
2020

Citation for published version (APA):
Beckmann, M., Lloyd, A. J., Wilson, T., Torres, D. P. M., Goios, A. C. L., Willis, N. D., Lyons, L., Phillips, H., Mathers, J. C., Nash, R. J., Sharp, H., & Draper, J. (2020). Calystegines are Potential Urine Biomarkers for Dietary Exposure to Potato Products. *Molecular Nutrition and Food Research*, *64*(20), [2000515]. https://doi.org/10.1002/mnfr.202000515

Molecular Nutrition & Food Research
www.mnf-journal.com

Calystegines are Potential Urine Biomarkers for Dietary Exposure to Potato Products

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Scope: Metabolites derived from specific foods present in urine samples can provide objective biomarkers of food intake (BFIs). This study investigated the possibility that calystegines (a class of iminosugars) may provide BIFs for potato (*Solanum tuberosum* L.) product exposure.
Methods and results: Calystegine content is examined in published data covering a wide range of potato cultivars. Rapid methods are developed for the quantification of calystegines in cooked potato products and human urine using triple quadrupole mass spectrometry. The potential of calystegines as BFIs for potato consumption is assessed in a controlled food intervention study in the United Kingdom and validated in an epidemiological study in Portugal. Calystegine concentrations are reproducibly above the quantification limit in first morning void urines the day after potato consumption, showing a good dose-response relationship, particularly for calystegine A_3. The design of the controlled intervention mimicks exposure to a typical UK diet and showed that neither differences in preparation/cooking method or influence of other foods in the diet has significant impact on biomarker performance. Calystegine biomarkers also perform well in the independent validation study.
Conclusion: It is concluded that calystegines have many of the characteristics needed to be considered as specific BFIs for potato product intake.

1. Introduction

After the major cereals rice, wheat and corn, potato tubers (*Solanum tuberosum* L.) are the most widely consumed starchy food internationally.[1] In addition, potato contributes significant amounts of vitamins (including vitamin C), minerals (including potassium), amino acids, and dietary fiber to many human diets. Although higher intakes of potatoes have been linked to increased rates of obesity, diabetes, and cardiovascular disease in some studies,[2] it is probable that any such associations were due to the fat added during cooking. Despite being a staple component of the diet in many countries globally, the impact of potato consumption on human health is relatively poorly understood.[2] Potato tubers contain a range of potentially toxic substances including glycoalkaloids and the starch in potatoes is largely indigestible without processing and cooking.[3] In addition to domestic processing including peeling, blanching, baking, and frying of raw tubers to produce chips (French fries), roasted potatoes, jacket potatoes, and boiled/mashed potatoes, a wide range of processed potato products are available commercially. Many of these processed foods are based on reconstituted potato and show considerable compositional differences from the raw tuber. Additionally, potato is a common component of ready-made complex meals. This diversity in uses of potato means that consumers often have difficulty quantifying accurately the amount of potato in their diet, making it challenging to link health outcomes to potato exposure.

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DOI: 10.1002/mnfr.202000515

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Dietary exposure can be estimated using self-reported measures such as food frequency questionnaires (FFQs), 24-h recall and diet diaries/records. However these methods have well understood limitations as a result of substantial misreporting and bias,[4] which is often exacerbated in those who are overweight or obese.[5,6] It has been demonstrated recently that metabolites derived from specific foods or food groups present in urine samples can provide biomarkers of dietary exposure.[7–11] Including measurement of biomarkers of food intake (BFIs)[12,13] could overcome some of the limitations of traditional dietary assessment methodologies by providing additional objective estimates of food exposure.[14]

A recent literature review exploring possible biomarkers of potato tuber intake[15] identified derivatives of polyphenol secondary metabolites (including chlorogenic acids) in urine following exposure to potato tuber products, but none could be considered specific to potato consumption. Similarly, a range of compounds resulting from the heating of potato products (including alkyl pyrazines, acrylamide and acrolein) were found in urine, but again a specific association with general potato consumption was considered unlikely. Several studies suggested that the well-known potato glycoalkaloids: alpha-solanine and alpha-chaconine, may provide more specific biomarkers of potato exposure. However, potato glycoalkaloid content was generally measured only in plasma or serum samples,[16–18] with no reports of their presence in urine. Although certainly specific to potato, the use of these compounds as general biomarkers of potato exposure may prove problematic as the glycoalkaloid content is confined largely to the skin and because concentrations increase dramatically after tuber "greening" caused by post-harvest exposure to light and wounding.[19,20] This characteristic distribution and response to environment means that consumption of potato-based foods that include the outer layers of the tuber will result in exposure to much higher levels of glycoalkaloids than that achieved after eating products derived from peeled potatoes. Furthermore, since higher concentrations of these toxins in food products is considered undesirable cultivar selection; agronomic practices, and storage and processing protocols are designed to lower the concentration of glycoalkaloids in potato-based food products.[19]

Calystegines are a family of nortropane alkaloids (a class of iminosugars) found largely in solanaceous plants,[20–23] where common forms include calystegines A_3 and B_2 (Data S1, Supporting Information). Calystegines were first detected in potatoes in the 1990s and, although not toxic to humans, may have potential to reduce glucose absorption resulting from inhibition of glycosidase enzymes.[24–26] Potato calystegines, in particular, may have benefits in stabilizing glucocerebrosidase which is deficient in Parkinson's and Gaucher's diseases.[27] Analytical methods for the determination of calystegines in plant-derived samples have been reviewed recently.[23] Because of their highly polar nature, earlier analytical approaches included a purification step using ion exchange columns followed by concentration by either freeze drying or rotary evaporation prior to derivatization and analysis by gas chromatography mass spectrometry (GC-MS). More recently, measurement of calystegines in plant extracts has been simplified by the use of liquid chromatography coupled to mass spectrometry (LC-MS) which avoids the need for purification and derivatization steps.[20,28] Concentrations of calystegines vary among potato cultivars and, although lacking extensive study, calystegines appear to be distributed relatively evenly throughout potato tubers but with slightly higher concentrations in the peel.[21,22,29,30] Unlike glycoalklaloids, calystegine metabolism is generally not influenced by postharvest exposure of potato tubers to wounding or light.[20]

To date, there are no reports of human food interventions that have studied the absorption, metabolism, and excretion of potato calystegines and so the utility of this class of compounds as potential urinary BFIs for potato tuber exposure has yet to be investigated. In the present study, we surveyed calystegines concentrations in a range of potato genotypes reported in previous studies. Rapid and sensitive micro-scale LC-MS/MS methods for the analysis of calystegines in cooked potato products and human urine have been developed and used to examine their potential as biomarkers of potato tuber consumption in both a controlled food intervention and an epidemiological study.

2. Results

2.1. Calystegine Content of Uncooked Potato Tubers

The impact of potato genetics on calystegine content was assessed by review of data reported in current literature (**Table 1**). The major iminosugars were found to be calystegine A_3 and calystegine B_2 in each potato cultivar tested when converted into the same format (mg kg^{-1} DW).[22,29,30] The estimated mean concentrations of total calystegines ranged from 150 to 475 mg kg^{-1} DW in studies carried out in different countries, with an average of content of 296 mg kg^{-1} DW.

2.2. Development of a Sensitive, Small-Scale LC-MS/MS Method for Quantifying Calystegines in Cooked Potato Products and Human Urine Based on Triple Quadrupole Technology

Due to their highly polar nature, the separation and detection of calystegines within mixtures of chemical standards was examined using hydrophilic interaction high pressure liquid chromatography mass spectrometry (HILIC-MS). Chromatographic behavior of calystegines A_3, B_1 and B_2 chemical standards showed good separation between A_3 and B_1/B_2 isomers although in all case the peaks were broad and showed evidence of tailing typical of HILIC-MS (Data S3A–C, Supporting Information). However, there were insufficient retention time differences between isomers B_1 and B_2 when applying specific MRM parameters for respective characteristic transitions Q1:176.081–Q3:112.185 and Q1:176.081–Q3:97.137 (**Table 2**, Data S3A–C, Supporting Information). Since calystegine B_2 is the most abundant of all B isomers, it was decided to add calystegine B_2 alongside A_3 to the calibration standards and use the strongest transitions Q1:176.081–Q3:140.191 as a common quantifier for potentially all calystegines B isomers (Data S3D,E, Supporting Information). Calibration curves for calystegine standards showed very good linearity in the concentration range from 0.0065 to 100 μg mL^{-1} (Data S4, Supporting Information). The limit-of-quantification (LOQ) and limit-of-detection (LOD)

Table 1. Calystegine content of raw tubers of different potato cultivars.

Study and analytical method	Cultivar	Total calystegines content in mature tubers [mg kg^{-1} DW[a)]]	Average calystegines content in mature tubers [mg kg^{-1} DW]
Kvasnicka et al. (2008)[30]	Ditta	498	
	Granola	399	
GC-MS	Karin	537	475
	Samanta	482	
	Magda	563	
	British Columbia Blue	374	
Griffiths et al. (2008)[29]	Alisa	212	
	Brodick	222	
GC-MS	Cara	277	236
	Maris Piper	136	
	Pentland Dell	333	
Friedmann et al. (2003)[22]	Atlantic	106	
	Dark Red Norland	34	
GC-MS	Ranger Russet	270	
	Red Lasoda	45	
	Russet Burbank	326	150
	Russet Norkota	88	
	Shepody	228	
	Snowden	102	
Peterson et al. (2013)[20]	Juliette	581	
LC-MS	Maris Bard	158	
	Princess	104	
	King Edward	248	
	Bintje	635	
	Marine	401	
	Asterix	374	323[a)]
	Folva	113	
	Sava	275	
	Terra Gold	576	
	Melody	221	
	Fontane	356	
	Desiree	162	
Average calystegine content of mature tubers = 296 mg kg^{-1} DW			

[a)] Assumes a conversion factor of 4.5 FW to DW in whole potato tubers. DW = dry weight.

for calystegines A_3 (0.0066 µg mL^{-1} and 0.0020 µg mL^{-1}, respectively) and B_2 (0.0219 µg mL^{-1} and 0.0066 µg mL^{-1}, respectively) were determined (Table 2).

Although there is substantial data on the calystegine content of raw potato tubers there is very little information on the impact of food preparation on calystegine levels. Samples of cooked potato products identical to those prepared by participants in the MAIN study[31] were assessed for calystegine A_3 and B isomer content by the above MRM methodology (**Figure 1**). The levels of both calystegine A_3 and B isomers in cooked intact tubers with skins on were at the higher end of the concentration range measured in fresh potato tubers, indicating minimal losses resulting from cooking. Processed potato products generally had a lower calystegine content, related to their lack of skin tissue and percentage tuber content.

The applicability of calystegine quantification was then tested using selected urine samples from previously reported studies with different levels of potato consumption.[32,31] Chromatographic behavior of calystegines A_3 and the mixture of B isomers in representative urine samples the day after consumption of varying portion size of potato are shown in **Figure 2**.

2.3. The Concentrations of Calystegines A_3 and B Isomers in Urine Are Related to Potato Exposure Level in an Acute Food Intervention

We have recently described a dietary exposure biomarker discovery and validation strategy based on a food intervention study involving free-living individuals preparing meals and collecting urine samples at home.[31] The study design took into account major sources of likely variance, including the impact of different food formulations and cooking methods and using meal patterns that mimic those encountered in a typical UK diet.[32] Analysis of the intervention menus[32] identified days on which the study cohort were exposed to meals containing potato products (Data S2, Supporting Information). In preliminary experiments a mastermix of FMV urine samples representing high exposure to potato products was analyzed by Triple Quadruple LC-MS/MS to determine which of the calystegines could be detected without inclusion of a urine fractionation or concentration step. Only calystegine A_3 and a peak representing a mixture of calystegine B isomers (predominantly calystegine B_2) were detected in diluted urine samples and thus further analysis was limited to these two target compounds using purified A_3 and B_2 as calibration standards. Calystegine A_3 was detected at levels ranging from 0.020 µg mL^{-1} to 2.75 µg mL^{-1} whilst the range of calystegine B isomers was slightly lower with a maximal concentration of 1.03 µg mL^{-1} (**Figure 3** and data shown in Data S5, Supporting Information). Urine concentrations correlated with potato exposure levels for both biomarkers, particularly calystegine A_3 (Figure 3; Spearman coefficient 0.64, p-value <0.001). Study participants were exposed to several types of potato products and cooking methods (microwave chips, boiled new potatoes, baked jacket potato, potato waffles, and oven chips). The average concentration of total calystegines in FMV urines was close to 0.004 µg mL^{-1} per gram of potato consumption for all potato-based food products (**Table 3**).

Low levels of calystegines have been reported in other solanaceous foods including tomato,[33] as well as aubergine and pepper[28] which might limit their use as a specific biomarker of potato exposure. Both tomato and pepper products were also consumed as components of the six menu plans of the MAIN study including a chicken tikka curry containing tomato puree, baked beans in a tomato sauce, fresh tomatoes, and beef Bolognese containing tinned tomatoes, and raw red and yellow pepper (Data S2, Supporting Information). There were no significant correlations between exposure levels of tomato or pepper and the

Table 2. TSQ-Ultra (QQQ) quantification parameters for calystegine in urine samples.

Food exposure class	Compound	tR	Ionization mode	Adduct	Parent (Q1) [*m/z*]	Tube lens voltage [V])		Quantification product (Q3) [*m/z*]	
Potato	Calystegine A_3	10.27	Pos	[M+H]1+	160.1	76		142.14	
Potato	Calystegine B isomers	10.69	Pos	[M+H]1+	176.081	76		140.084	
Food exposure class	Compound	SRM-Quan	SRM-Qual (1)	SRM-Qual (2)	CE-Quan	CE-Qual (1)	CE-Qual (2)	LOD [μg mL^{-1}]	LOQ [μg mL^{-1}]
Potato	Calystegine A_3	142.14	125.12	79.31	11	15	23	0.00197	0.00656
Potato	Calystegine B isomers	140.084	112.185	158.047	14	16	12	0.00656	0.02187

tR, retention time; Q1, parent *m/z*; Q3, quantification product *m/z*; SRM, selected reaction monitoring; LOD, limit-of-detection; LOQ, limit-of-quantification; SRM-Quan, quantification transition; SRM-Qual (1), qualifier transition 1; SRM-Qual (2), qualifier transition 2; CE-Quan, collision energy for quantification transition; CE-Qual (1), collision energy for qualifier transition (1); CE-Qual (2), collision energy for qualifier transition (2).

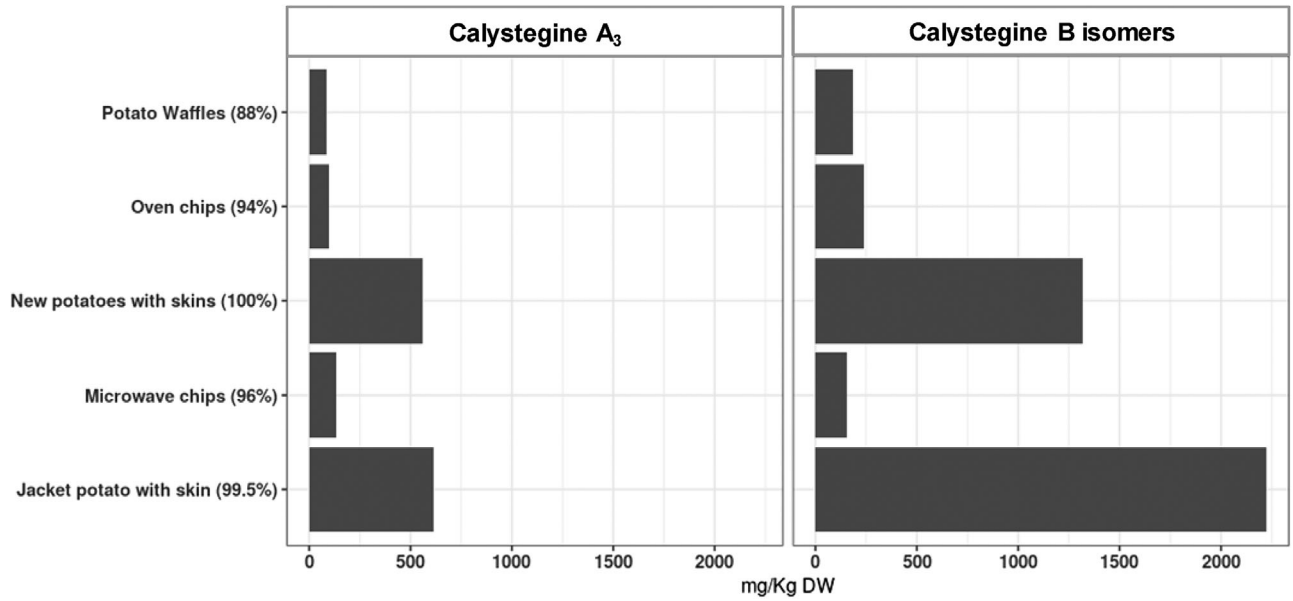

Figure 1. Calystegine levels in cooked potato products measured by LC-MS. Numbers in brackets indicate the percentage content of raw potato tuber in each potato product.

Table 3. Calystegine concentration in FMV urine after consumption of different potato products.

Potato product on test day	Microwave chips	Boiled new potatoes	Oven chips	Jacket potatoes and waffles
Amount consumed [gm]	100	200	170	315
Average total calystegine concentration in urine [μg mL^{-1}] per gram potato product consumed	0.0043	0.0043	0.0037	0.0039

concentrations in urine for either calystegine biomarker (Data S6A, Supporting Information).

2.4. The Excretion Kinetics of Calystegines

To assess the utility of calystegines as potential biomarkers of potato product exposure an acute intervention study was undertaken using microwaved jacket potatoes as a representative food (**Figure 4**). FMV urine samples from participants that had avoided consuming potato-containing food products for 48 h contained very low levels of both calystegines. Analysis of postprandial urines revealed a steady increase in the concentration of both calystegines with time during the intervention day with levels still elevated in FMV the next day. Calystegine levels were higher in FMV urine samples on the intervention day from participants who had only avoided potato-containing foods for 24 h.

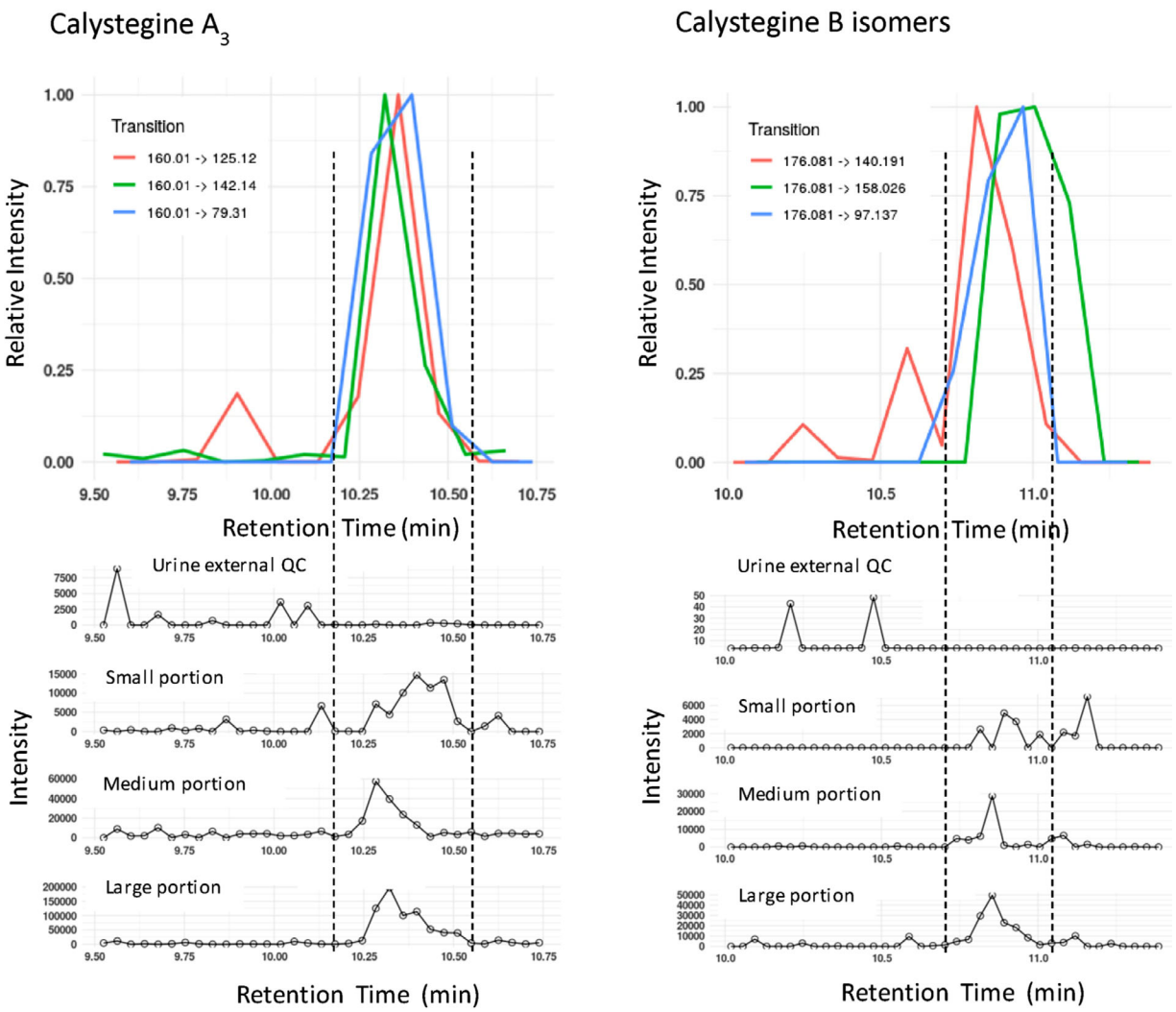

Figure 2. Multiple reaction monitoring (MRM) chromatograms of calystegines A_3 and B isomers in urine samples and controls. The top half of the figure show reference quantifier and qualifier peaks for both calystegines in an example urine sample collected after consumption of a large portion of potato. All of the transition chromatograms are scaled to relative intensity (calystegine A_3, product masses *m/z* 79.31, 125.12, and 142.14 and calystegine B isomers, product masses *m/z* 97.137, 140.191, 158.026). The lower half of the figure shows quantifier peaks in first morning void (FMV) urines after consuming large, medium, and small portions of potato in an intervention study. A blank quality control (external QC) urine sample is included. The reference transition peaks are aligned to quantifier chromatograms in the lower half of the figure and the vertical black dotted lines indicate the retention time windows used to calculate peak areas.

2.5. Calystegines Are Potential Biomarkers of Potato Product Exposure in a National Survey of Nutritional Consumption and Physical Activity

Study participants ($n = 95$) in the IAN-AF study[34] were assigned into four potato consumption categories (see Data S7, Supporting Information: none, $n = 26$; small, $n = 32$; medium, $n = 18$; and large, $n = 19$) using the FSA "food portion sizes" guide) based on 24 h dietary data. Calystegine A_3 was present at approximately twice the concentration of Calystegine B isomers in all samples (Data S7, Supporting Information). The concentration of calystegine A_3 measured in FMV urine samples correlated with exposure level of potato products (Spearman = 0.46; *p*-value 0.003: **Figure 5**). The same participants were also assigned to exposure groups in relation to tomato, pepper and aubergine products (none, small, large) and urine samples screened for the presence of calystegines (Data S6B, Supporting Information). Unlike for potato exposure, there was no significant relationship between the levels of tomato, pepper, and aubergine consumption and the levels of calystegines in the urine.

3. Discussion

3.1. Impact of Genotype on Calystegine Content of Potato Tubers

For calystegines to have value as BFIs for potato it is important to understand the likely impact of the source of potato on calystegine content. The methods of reporting the quantity of calystegines in potato tubers differs considerably between investigations. For example, from a sampling perspective, some

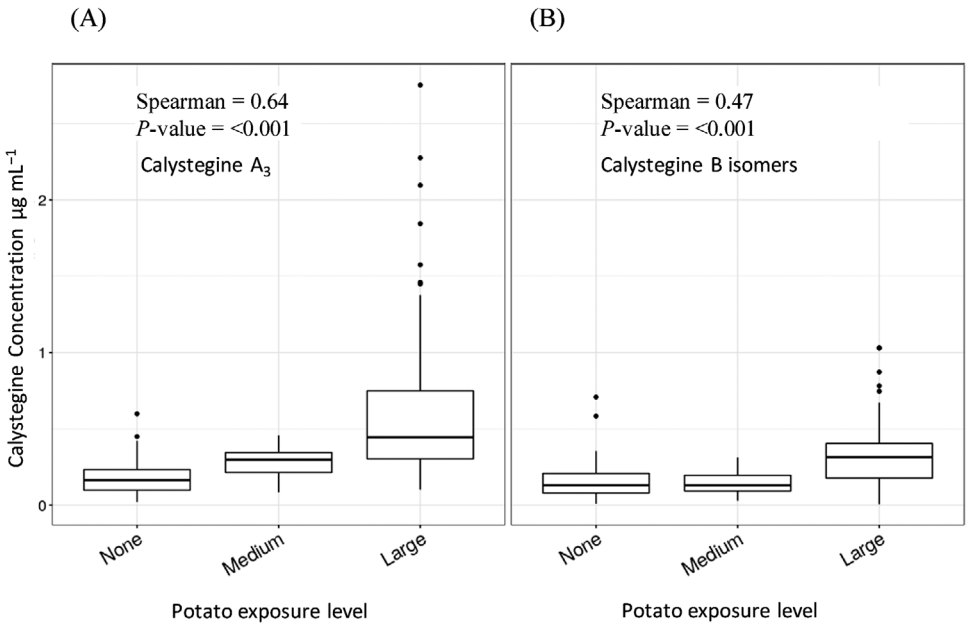

Figure 3. Calystegine concentrations in first morning void (FMV) urine following differential acute exposure to dietary potato products. A) Calystegine A_3; B) calystegine B isomers.

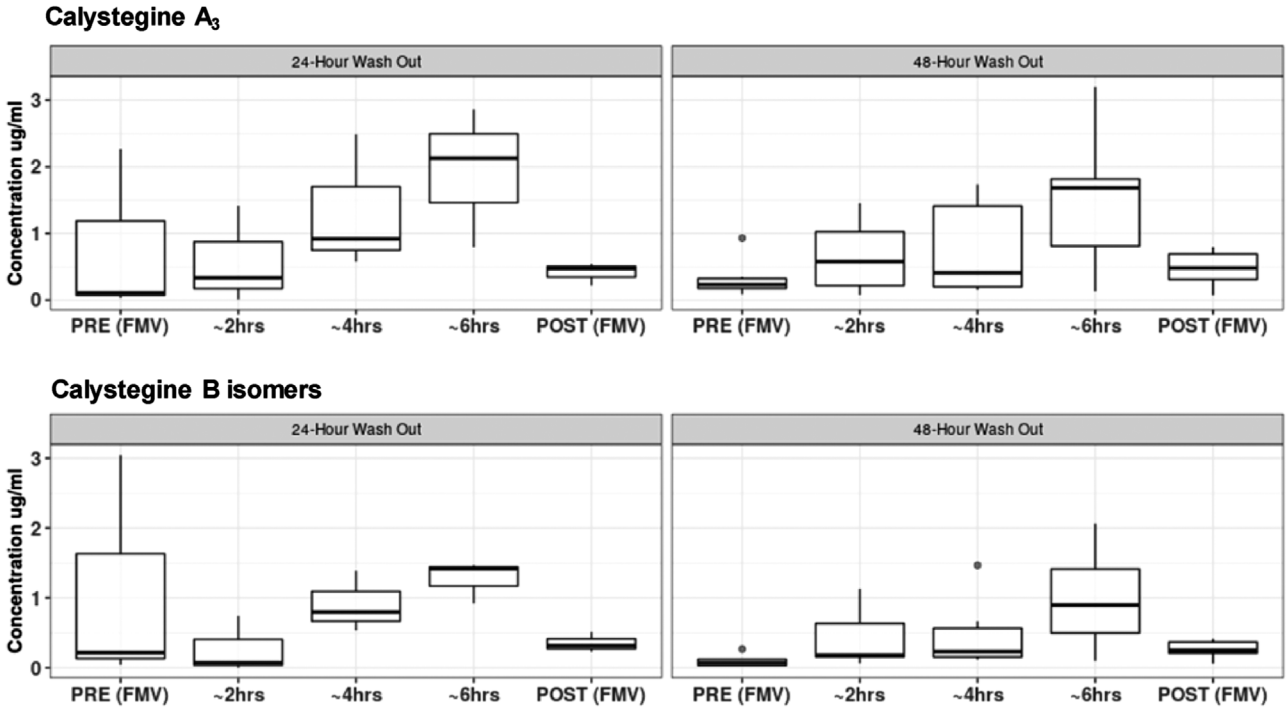

Figure 4. Excretion kinetics of calystegines after consumption of a jacket potato. Box plots show calystegine levels in urine samples taken before and at specific time points after the intervention. Participants are grouped by the length of the wash-out period (24 vs 48 h). PRE (FMV), first morning void urine on the day of intervention; POST (FMV), first morning void urine on the day after intervention.

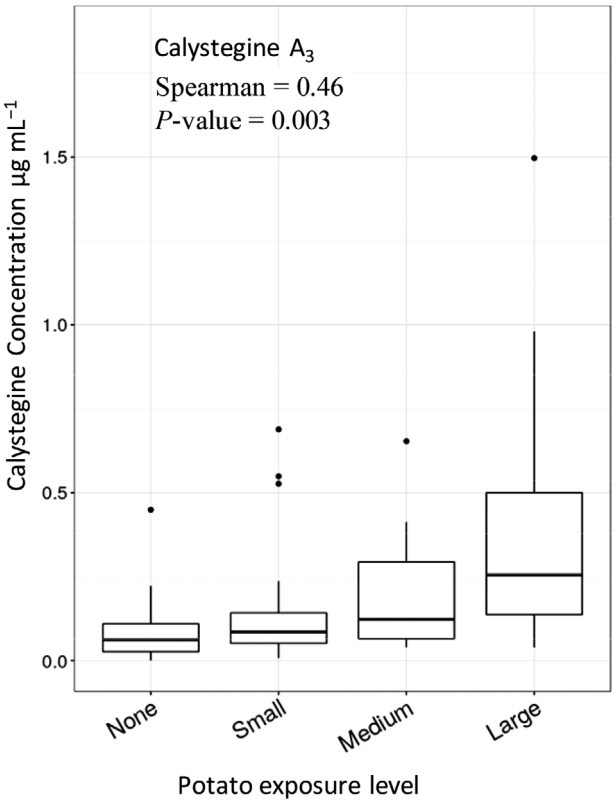

Figure 5. Calystegine A_3 concentration in first morning void (FMV) urine following differential exposure to dietary potato products in an epidemiological study.

reports provide quantities for all individual calystegine isomers (e.g., ref. [28]) in fresh uncooked tubers from a wide variety of international sources, but provide no information with regard to genotype. Other researchers[20] have measured the concentration of just the most abundant potato calystegines (A_3 and B_2 and B_4) in fresh uncooked tubers and provide data on inter-cultivar variability. The concentrations of the main potato calystegines (A_3 and B_2) in freeze-dried uncooked tuber tissue from specific cultivars has also been reported.[22,29,30] When data are expressed on the same basis, the estimated mean concentrations of total calystegines range from 150 to 475 mg kg^{-1} DW in studies carried out in different countries (Table 1). As there is no overlap in the potato genotypes selected for inclusion in each of these studies the origin of this diversity is difficult to evaluate but may reflect differences in measurement methods, or effects of climate or agronomic practices or simply to the differences in cultivar preferences in individual countries. Previous reports have suggested that tuber peel contained higher concentrations of calystegine compared to medulla and cortical tissues; which may be related to the retention of "sprout" tissues in these samples.[21,22,29,30] Although the present data revealed that neither food processing or cooking methods significantly altered calystegine levels in potato-containing food products, it was noticable that calystegine concentrations were higher in foods which retained tuber skins (Figure 1). Preliminary analysis of early-harvested "salad" potato varieties (Gemson and Belina) which are normally consumed without peeling contained significantly higher concentrations of calystegines (data not shown).

Consistent with the present data, the majority of studies showed that the most abundant calystegine in potato tubers, across all cultivars, is B_2 followed by A_3, with ratios of B_2/A_3 reported to range from 1.7 to 6.4 in the United States[22] to 0.9–2.3 in the United Kingdom.[29] In contrast, in some studies calystegine A_3 appeared more abundant than B_2 in potatoes.[20,28] Although these latter studies both used LC-MS analysis,[20,28] rather than more widely used GC-MS technology, these differences are more likely to be related to the methodology used to extract potato tissues (Data S8, Supporting Information). From a physicochemical perspective, A_3 contains only three hydroxyl groups as compared to four hydroxyl groups in B_2 so that the polarity of the extraction medium is likely to affect the relative efficiency of extraction of the different isomers. Although this requires more detailed investigation, it would appear that the ratio of B_2/A_3 recovered after extraction increases from acetonitrile, to methanol and to water-based extraction, resulting in a trend for calystegine A_3 content to decrease and calystegine B_2 to increase (Data S8, Supporting Information). Against this background of different measurement methodology, it remains a possibility that the ratio of specific calystegines may differ systematically between genotypes. Although calystegine A_3 on its own performed well as a biomarker of potato intake in the United Kingdom and Portuguese studies, we suggest it will be prudent to explore also the performance of total calystegines content to validate the future utility of calystegines as potato tuber exposure biomarkers in other populations.

3.2. Measurement of Calystegines in Urine Samples

Highly polar compounds such as calystegines are not well separated by conventional reverse phase chromatography, but hydrophilic LC-MS (HILIC) methods have proved successful in doing so (reviewed by Romera-Torres et al.[23]). Good chromatographic separation of calystegines A, B, and C isomers from an extract of potato tubers was demonstrated utilizing a Luna NH2 HILIC column but all four calystegine B isomers coeluted in a single peak.[33] We have reported recently the simultaneous measurement of multiple dietary exposure biomarkers in human urine samples using a combination of reverse phase and HILIC chromatography.[32,31,35] Using a similar p-HILIC column (ZIC) to measure calystegines in diluted urine samples, we demonstrated that comparable characteristics were evident in terms of elution order and separability. Importantly, routine quantification was achieved without interference with the measurement of other targeted dietary exposure biomarkers. Since calystegines A_3 and B_2 are the main isomers found in potatoes, a lower chromatographic resolution resulting in only two respective peaks (A_3 and B isomer calystegines) for routine biomarker quantification on ZIC p-HILIC should be adequate.

3.3. Performance of Calystegines as Specific Urine Biomarkers of Potato Tuber Dietary Exposure

Calystegines A_3 and B isomers on regression analysis showed a linear relationship between LC-MS signal and concentration

levels with LOQs of, respectively, 0.0066 and 0.021 μg mL^{-1} when measured in standard solutions. In a controlled intervention study, the lowest calystegine A_3 concentrations observed in urine collected the day after potato consumption were all at least ×10 fold greater than the LOQ. In addition, although generally present at lower concentrations than for A_3, the concentrations of B isomers were all in the quantifiable range (Data S5, Supporting Information and Figure 3). Examination of excretion kinetics demonstrated a steady rise during the day of consumption and significant carry over of calystegine from one day to the next (Figure 4), suggesting that both post-prandial and FMV urine samples were suitable for biomarker measurement. Additionally, the study design[31] allowed a direct comparison of calystegine concentrations in FMV urine after consumption of three different potato products all consumed at the same meal time on different days (Data S5, Supporting Information and Table 3) in either medium (microwave chips) or large (oven chips or boiled potatoes) portion sizes. When expressed as the amount of total calystegines per milliliter of FMV urine per gram of potato product consumed the previous day, the values for all three different potato formulations was on average 0.004 μg mL^{-1}. It should be noted that potato consumption was not always excluded in the MAIN study on the day prior to the potato product intervention (Data S5, Supporting Information). However, on day 1 during the dietary intervention study (Menu 5, Data S2, Supporting Information), participants consumed potato products twice during the day (potato waffles at breakfast and jacket potatoes at dinner), but yet again the average level of total calystegines in the next day FMV urine per gram of potato product was 0.004 μg mL^{-1} (Table 3).

We suggest that these data highlight the value of using urine samples from the MAIN study[31] (which was designed to mimic a typical UK diet) in combination with the quantitative measurement simultaneously of a panel of biomarkers to validate the real-world performance of candidate BFIs.[12,13]

There is evidence that peel samples from uncooked mature potatoes may contain higher concentrations of calystegines than in the cortex and medulla regions of the tuber.[29,30] The present data support this suggestion. However, in the present study, we did not find that consumption of cooked potato products containing peel (such as the new potatoes and jacket potatoes) resulted in significantly higher calystegine levels in FMV urine (Table 3). These observations indicate that differences in preparation/cooking method or assay sensitivity should have little impact on biomarker performance. Indeed, with correlation coefficients close to, or greater than 0.5 (Figure 3), the dose response between quantity of potato consumed and calystegine concentrations in FMV urine are good. The Portuguese epidemiological study pro vided an opportunity to examine dose responsiveness in relation to self-reported estimates (24 h, multiple pass dietary recall) of potato intake at an even a wider range of consumption levels (Figure 5) and again calystegine A_3 performed well.

A noticeable trend in the intervention study was the number of outliers outside of the top quartile in the "large" potato portion size intervention group (Figure 2). In addition, the mean calystegine concentration in FMV urine of participants consuming a "large" portion of potato products was higher in the intervention group (≈0.4 μg mL^{-1}) than in the epidemiological survey (≈0.25 μg mL^{-1}). These differences could reflect the fact that prescribed amounts of potato were consumed in the intervention study whereas, in the observational study, participants over-estimated their intake of potato products. Further investigation (Supplemental data 3) revealed that the majority of participants classified as "large" consumers of potato products in the intervention study also reported consuming potato products on the day before the intervention. These data again confirm that ingested calystegines (A_3 in particular) might not be fully excreted until at least 24–36 h after consumption. Such potential sequestration of calystegines within the tissues leading to slower elimination from the body would be a useful property for a biomarker of habitual exposure in studies limited to collection of FMV spot urine samples. Further support for this suggestion arises from the fact that baseline concentrations of calystegine in participants classified as "none" fell into a range that overlapped with that found in participants classified as "medium" level consumers. Again dietary records showed that many of these individuals had eaten potato products the previous day. Since food products and complex meals containing potatoes are a very common part of the UK diet, most individuals will have multiple exposures to potato during a typical week and this fact will need to be taken into account when considering the use of calystegines as a BFI in relation to criteria suggested recently.[13]

3.4. Specificity of Calystegines as Potato Tuber Biomarkers

Several studies have reported the presence of calystegines in other solanaceous foods in addition to potato including, aubergine, bell peppers, and tomato.[26,28,33] There is also recent reports of calystegines in the Brassicacae,[36] but not in any food species. Although calystegines found in urine may also reflect exposure to other types of food products, there are several lines of evidence that suggest they are relatively specific to potato product intake in the present study. In terms of overall contribution, it has been reported the mean content of calystegines in potatoes to be at least 8× higher than that reported for aubergine and in the limited number of bell peppers analyzed, only trace amounts of calystegines were detected.[28] Similarly, calystegines have been detected in fresh tomato and tomato products, but levels were very low.[33] In the present study, we did not find a positive correlation between exposure to tomato, bell pepper, or aubergines and the calystegine content of urine. In relation to habitual diet, the relative contribution of different foods to the calystegine content of urine will also be related to the frequency of exposure to the different foods. Data from the UK National Diet and Nutrition Survey (NDNS) Years 1–3[37] revealed that potato was consumed by 2847 of the 3073 volunteers (93%), tomato (including puree) was consumed by 99% of the volunteers, bell peppers were consumed by 28% of the volunteers and aubergine only 1%. For the UK population, the majority of any calystegine signal in urine will likely be derived from potato exposure.

4. Concluding Remarks

A scheme of factors to be considered for the critical assessment of BFI has been outlined recently.[13] The present data suggest that calystegines, and particularly calystegine A_3, score highly

Table 4. Validation characteristics of calystegines as biomarker of potato intake using the scheme devised for biomarkers of food intake (BFI's) critical assessment.[13]

Plausibility	Calystegines occur at high levels in potato tubers and although not absolutely specific are much higher in potato than other foods, such as aubergine, which are eaten much less frequently.
Dose-response	We show a good dose-response relationship in a controlled intervention study at different intake levels of foods in the context of normal (complex) meals and eating patterns.
Time-response	Excretion kinetics demonstrate that urine samples taken either late on the day of consumption or as a first morning void will be suitable for calystegine quantification. Calystegine levels are almost an order of magnitude above the quantification limit in first morning void urines in both interventional and epidemiological studies. There appears to be no metabolism of calystegines (searched for glucuronide and sulfate biotransformation products in LC-MS data).
Robustness	We have recently described a dietary intervention study designed specifically to mimic the eating habits of the UK population[32,31]; participants were provided with food products and four day menus to allow them to prepare and consume meals in their own homes in the context of a normal UK eating pattern (breakfast, lunch, dinner). Calystegine levels in urine reported the intake of potato products when presented in different formulations and using different cooking methods that are commonly encountered in the United Kingdom. The levels of calystegines in potentially confounding foods (such as aubergine) are much lower and they are very infrequently consumed in comparison.
Reliability	Calystegine biomarkers compare well with both menu composition and eating compliance records in an intervention study and align well with 24 h recall dietary data in an epidemiological study.
Stability	Calystegines seem largely unaffected by a range of food processing and cooking methods. Calystegines, like the majority of imino-sugars appear very stable in urine samples after storage times of 5 years or more.
Analytical performance and reproducibility	Calystegine measurement by HILIC LC-MS has been shown to be feasible as part of a complex panel of food intake biomarkers in present study. Long term analytical performance and reproducibility is currently being assessed in several cohort studies.

on many validation characteristics (**Table 4**) and should be considered as strong candidates for biomarkers of potato product intake.

4. Experimental Section

Ethical Approval: The MAIN Study was approved by the East Midlands - Nottingham 1 National Research Ethics Committee (14/EM/0040). For the potato biomarker kinetics study, a favorable ethical opinion was obtained following review by the Aberystwyth University Research Ethics Panel. For the epidemiological study, ethical approval was obtained from the National Commission for Data Protection, the Ethical Committee of the Institute of Public Health of the University of Porto and from the Ethical Commissions of each one of the Regional Administrations of Health. All participants gave written informed consent, and the study was carried out in accordance with the Declaration of Helsinki.

Food Intervention Study Design and Urine Sampling: The MAIN (Metabolomics at Aberystwyth, Imperial and Newcastle) Study at Newcastle included two controlled food intervention studies in free-living populations who consumed the test foods as part of two 3-day menu plans, equating to six different menus (experimental period 1, $n = 15$ where 53% female, all non-smokers, age range = 22 to 63; experimental period 2, $n = 36$ where 58% female, all non-smokers, age range = 19 to 77 years).[32,31] Multiple potato products were consumed over the six different menus[31] including, microwaved chips (100 g), new potatoes boiled with skins (200 g), potato waffles (116 g), baked jacket potato (200 g), and oven chips (170 g) (Data S2, Supporting Information). The portion sizes of the selected potato products were defined as small, medium, or large with reference to the Food Standards Agency (FSA) "food portion sizes" guide. A "large" portion was defined as >1.5 times the FSA medium portion size and a "small" portion size as <0.5 times the FSA medium portion size.[38]

Urine sampling methods were implemented based on the previous studies[39,40] and participants were asked to collect a series of urine samples including the first morning void (FMV) the day after each menu plan.

Potato Consumption Biomarker Excretion Kinetics: Participants ($n = 7$, 29% female, all non-smokers, age range = 25 to 66) were recruited and asked to continue consuming their habitual diet, but to omit potato containing products for 24–48 h prior to the study day. On the intervention day after this washout period, they were asked to collect a first morning void (FMV) urine at home and store at 4 °C. Participants consumed a packed lunch consisting of a jacket potato (200 g), baked beans, mixed leaf salad, banana, and diet lemonade. They then collected post-prandial urine samples every 2 h until bed-time and a FMV urine sample the next day. Participants were allowed to consume a meal lacking potatoes of their own choice on the evening of the intervention day if desired.

Urine Sample Preparation: Urine samples were prepared and adjusted as previously reported.[32,31] In brief, all urine samples were normalized by refractive index prior to analysis to account for differences in fluid intake by participants and to ensure all that all MS measurements were made within a similar dynamic range within the linear range of the instrument. Samples were defrosted overnight in a 4 °C fridge, centrifuged (600 × *g* for 5 min at 4 °C), placed on ice and aliquots of thawed urine (1000 µL) was transferred into labeled 2 mL Eppendorf tubes. The remaining samples were returned to a −20 °C freezer. An OPTI Hand Held Refractometer (Bellingham Stanley Brix 54 Model) was used to record the specific gravity (SG). Based on these figures, aliquots of the required amounts of urine from centrifuged 2 mL Eppendorf tubes and ultra-pure (18.2 Ω) H_2O were transferred into new tubes for extraction; this ensured that all samples had the same refractive index.

Epidemiological Study and Urine Sampling: The study participants ($n = 95$) were volunteers who participated in the Portuguese National Food, Nutrition and Physical Activity Survey (IAN-AF), whose aims and methods were described previously.[34] A 24 h dietary record was collected by trained nutritionists using the "eAT24" Software which facilitates the assessment of dietary data using an automated multiple-pass method (five steps).[41] All foods, including beverages and dietary supplements, consumed were recorded per eating occasion and quantified and described as eaten. A recipe module was also created, in which the recipes were disaggregated into raw ingredients allowing the description and quantification of each item including potato). A FMV urine sample was collected in a 500 mL container and kept refrigerated (4 °C). Samples were aliquoted: 1 × 45 mL (in 50 mL Falcon pre-labeled tube) + 10 × 1.5 mL (in 2 mL pre-labeled microtubes). These aliquots were refrigerated immediately before being moved to −80 °C storage, within 24 h, until analysis.

Chemicals and Reagents for LC-MS/MS: Methanol (primer trace analysis grade, Fisher Scientific, UK) was used for urine extraction and standards preparation. Acetonitrile (Optima LC/MS grade, Fisher Scientific, UK), methanol (HPLC grade, Fisher Scientific, UK), and ammonium acetate (Optima LC-MS grade, Fisher Scientific, Belgium) were used for preparing the LC mobile phase. Water was ultra-pure water (18.2 Ω) drawn from an Elga Purelab flex water purifier system (Taiwan). Chloroform (for HPLC stabilized with Amylene, Fisher Scientific, UK) was used for potato product extraction. Calystegines A_3, B_1, and B_2 standards were provided by PhytoQuest (Aberystwyth, UK) at 95% GC-MS purity.

Potato Product Cooking and Sample Preparation for LC-MS/MS: The potato products for calystegine analysis that were consumed in the intervention study (Section 2.2) were cooked in a domestic kitchen as described previously.[31] Briefly, the microwave chips were microwaved at full power from frozen for 3 min. New potatoes were cooked by boiling in tap water for 15 min until soft using the cooker hob, leaving skins on. Potato waffles were oven baked (220 °C) from frozen until crisp and golden. The jacket potatoes were microwaved at full power from frozen for 5 min. The oven chips were oven cooked (220 °C) from frozen until crisp and golden. After allowing to cool samples of potato products were stored at −20 °C in 50 mL falcon tubes; for both jacket potatoes and boiled new potatoes the potato peel comprised at least 30% of the samples. The % dry weight of each potato product was determined after freeze drying. For each potato product ≈100–200 mg of frozen tissue were placed into 2 mL Eppendorf tubes (×3 replicates) containing a 4 mm steel ball bearing, then immediately flash frozen. The final weight of each sample was recorded. Batches of samples were processed in a Mixer Mill MM 301 (Retsch GmbH) for 30 s at 30 Hz, then 1 mL of extraction solvent (2:5:2 chloroform:methanol:ultra-pure water) added to each tube. Samples were vortexed for 10 s and then then agitated at 1400 rpm, at 4 °C, for 20 min to extract metabolites. A further 150 µL of methanol was added to each tube (to avoid phase separation) which was then vortexed and centrifuged at 13 000 rpm, for 6 min at 4 °C. 50 µL of the supernatant was then diluted with 450 µL of 70:30 ultra-pure water:methanol containing 0.1% formic acid, and 5.55 µg mL^{-1} of 4-chloro-DL-phenylalanine as an internal standard. The micro-extraction method was validated by determining the recovery of calystegines after successive extractions of cooked potato products and subsequent quantification of calystegines. 90% of total extractable calystegines were obtained after the first extraction; a further two extractions led to an increase in 9% of total calystegine content. All calystegine measurements were made on the first extract and subsequently data were corrected for recovery.

Development of Methodology for Quantification of Calystegine Content in Urine and Cooked Potato Products: Urine samples were prepared and analyzed as reported previously.[31] Briefly, specific gravity adjusted urine samples were diluted 1 in 10 with water:methanol (70:30, v:v) containing 0.1% formic acid and 5.55 µg mL^{-1} of 4-chloro-DL-phenylalanine as an internal standard. Extracts of cooked potato products were prepared as outlined in 2.9. Quantification of calystegines was performed on a TSQ Quantum Ultra triple quadrupole (QQQ) mass spectrometer (Thermo Scientific), equipped with a heated electro-spray ionization (HESI) source and coupled to an Accela UHPLC system (Thermo Scientific). Hydrophilic interaction liquid chromatography (HILIC) chromatographic separation was achieved on a ZIC-pHILIC column (polymeric 5 µm, 150 × 4.6 mm, Merck). The mobile phase consisted of A) 10 mm ammonium acetate in water:acetonitrile (95:5) and B) 10 mm ammonium acetate in water:acetonitrile (5:95). A linear gradient program was used from 95% B to 20% B in 15 min at a flow rate of 400 µL min^{-1}. The solvent flow was increased at 15.01 min to 500 µL min^{-1} and held constant for 5 min. The column was then equilibrated at 95% B for 5 min. Column oven and autosampler tray were maintained at 60 and 14 °C, respectively. To ensure consistent sample delivery, 20 µL were injected using a 20 µL loop and partial loop injection mode. After each injection, the syringe and injector were cleaned using a 10% HPLC grade MeOH solution in ultra-pure water (1 mL flush volume) to avoid sample carryover. Spectra were collected at a scan speed of 0.010 s. A scan width of 0.010 m/z, and peak width (Q1, Q3) of 0.7 FWHM were used. Mass spectra were acquired in multiple reaction monitoring (MRM) mode, in positive and negative ionization mode simultaneously. MRM transition as well as values for shimmer offset, collision energy, and tube lens were optimized automatically using Quantum Tune Master (V1.5SP1, Thermo Scientific).

Absolute concentrations were calculated using a nine-point calibration curve (0.006561–100 µg mL^{-1}) for each calystegine. Xcalibur (V3.0.63, Thermo Fisher Scientific Inc.) was used for peak integration, calibration, signal-to-noise estimation, and quantification. Raw files (ThermoFisher) were converted to mzML[42] using msconvert in the ProteoWizard tool kit.[43] Briefly, selected reaction monitoring (SRM) chromatograms were extracted using the R library mzR and peak areas were calculated by extracting pre-defined retention time windows (based on calibration standards) around the peak apex. For each calibration standard, a quadratic equation was used to model the relationship between peak area and concentration. A squared fit of log10-transformed peak area values accommodated best the wide concentration range for biomarkers in high and low consumers, without compromising accuracy and normal distribution requirements for regression analysis. Reproducibility of measurement was determined using the relative standard deviation (RSD) of a multicomponent calibration standard containing calystegines and an external urine QC sample using a "master mix" of pooled samples as previously reported.[32,31] Each daily batch of samples subjected to MRM analysis contained 15–20% calibration and QC samples.

The limit of detection (LOD) and limit of quantification (LOQ) of calystegines A_3 and B_2 within chemical standard mixes were calculated as the lowest concentration of each biomarker giving a signal-to-noise ratio of 3:1 and 10:1, respectively.

Correlations between portion size and biomarker concentration in urine were estimated using Spearman's rank correlation. Students *t*-test was applied to the highest and lowest portions and biomarker concentration. In all instances, values were first log10 transformed.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors thank the participants in Newcastle and Portugal for their commitment in the food intervention and dietary survey studies, respectively. The researchers acknowledge all the institutions and persons that integrated the activity of the IAN-AF (2015-2016) Consortium at all phases of the Survey. The researchers thank Puffin Produce Ltd. for providing potato tubers.

Author Contributions

The authors' contributions to the work were as follows: M.B. developed quantification methods, supervised MS support staff, wrote the manuscript; A.J.L. produced figures, researched literature, and wrote the manuscript; T.W. developed quantification methods, and wrote the manuscript; D.T. and A.G. undertook volunteer recruitment in Portugal, coordinated volunteer visits, and supervised support staff; N.D.W., undertook volunteer recruitment in Newcastle, coordinated volunteer CRF visits, and supervised CRF support staff; L.L. and H.P. performed QQQ technical support and data generation; J.C.M. coordinated project, supervised research in Newcastle University; R.J.N. and H.S. generated calystegine standards and provided unpublished data on GC-MS analysis in potato tubers; J.D. coordinated project, supervised research in Aberystwyth, designed figures, and wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

calystegines, solanaceous, potato exposure biomarker, biomarkers of food intake, metabolomics

Received: May 28, 2020
Revised: June 25, 2020
Published online:

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